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Measurements of [Ca^{2+}] using fura-2 in glioma C6 cells expressing calretinin with GFP as a marker of transfection: no Ca^{2+}-buffering provided by calretinin

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Abstract

Glioma C6 cells were transfected with a plasmid containing the calretinin (CR) and green fluorescent protein (GFP) coding regions to analyze the effect of CR's presence on $[Ca^{2+}]_i$. Positive transfectants were identified by the detection of GFP and $[Ca^{2+}]_i$ was measured using fura-2 as a probe. We found that neither the basic $[Ca^{2+}]_i$ nor activated $[Ca^{2+}]_i$ achieved by exposure to ionomycin, ADP or thapsigargin were affected by CR's presence in transfected cells, despite the ability of CR to bind Ca^{2+} as part of fusion protein. The level of expressed CR was estimated as at least 1 μM. The presented results suggest that CR's function is unlikely to be an intracellular Ca^{2+}-buffer and support the hypothesis that CR might be involved in a specific Ca^{2+}-dependent process. The results of this work also show that the S65T mutant of GFP is compatible with fura-2 measurements of intracellular $[Ca^{2+}]$. We have demonstrated that the presence of GFP, as a transfection marker of glioma C6 cells, does not disturb fura-2 fluorescence, the basal or activated $[Ca^{2+}]_i$ in these cells.

Keywords: Calretinin; $[Ca^{2+}]$ imaging; Green fluorescent protein; Transfection

1. Introduction

Ca^{2+} plays a crucial regulatory role in eukaryotic cells and its concentration must be strictly regulated. Calcium binding proteins (CaBPs) are necessary to allow Ca^{2+} to induce many intracellular processes.

Only a few of the known CaBPs have a well defined function, for example calmodulin and troponin C, but the role of most CaBPs is still not fully understood. Calretinin (CR) is a calcium binding protein homologous to calbindin D28k (58% identical residues) consisting of 6 EF-hand calcium binding motifs [1–3]. CR is mostly present in the cytosol [4,5] but it is also present in membrane fractions [5,6]. CR is generally expressed in neurons [1,7] and is also found in some cancer cell lines [8,9]. The function of CR is not yet understood and so far, results

Abbreviations: CR, calretinin; GFP, green fluorescent protein; CMV, cytomegalovirus promoter; $[Ca^{2+}]_i$, intracellular calcium concentration; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; SDS, sodium dodecyl sulfate; G-418, geneticin; AM, acetoxymethyl ester; BSA, bovine serum albumin; MEM, minimal essential medium; HCV, human urothelial cell

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have been published that provide an inconsistent picture. Some evidence suggest that the possible function of CR is the buffering of intracellular [Ca^{2+}] which protects cells against Ca^{2+}-overload [10–16]. However, it has been shown that CR changes its conformation upon Ca^{2+} binding with similar characteristics as calmodulin, thus behaving like a typical Ca^{2+} sensor [17–19]. Also, there are data indicating that the presence of CR in neurons does not provide them with a greater resistance to Ca^{2+}-overload [20–23]. When PC12 cells were transfected with plasmids to express CR they did not achieve a stronger resistance to Ca^{2+}-overload compared to the total population of the studied cells [24]. The aim of the present studies was to explain the latter results and to shed more light on possible CR functions, and check whether CR may act as a Ca^{2+}-buffer. We analyzed the $[Ca^{2+}]_i$ before and after cell activation in cells differing in CR expression levels resulting from transfection. The cells expressing CR were identified by GFP fluorescence, whereas $[Ca^{2+}]_i$ was measured using fura-2. The $[Ca^{2+}]_i$ was simultaneously analyzed in transfected cells showing GFP fluorescence and untransfected cells showing no GFP fluorescence.

2. Materials and methods

2.1. Chemicals

Minimal essential medium (MEM), newborn calf serum, fetal calf serum, trypsin-EDTA 1×solution and lipofectamine reagent were purchased from Gibco BRL; G-418, thapsigargin, ionomycin, 4-chloro-1-naphthol and anti-rabbit IgG conjugated with horseradish peroxidase were purchased from Sigma; the green fluorescent protein vector (mutant S65T, which has a single excitation peak at approximately 490 nm, and several fold more intense fluorescence than wild-type GFP) was from Clontech; fura-2 AM was obtained Molecular Probes and ADP from Serva; low molecular mass standards were from Pharmacia, and the Chemiluminescent Substrate kit came from KPL (Kirkegaard and Perry Laboratories).

2.2. Cell culture

Glioma C6 cells were cultured in Hank's MEM supplemented with 7% (v/v) newborn calf serum, penicillin (50 IU/ml) and streptomycin (50 μg/ml). The media was changed every 2–3 days and cells were passaged when confluent. Cultures were maintained in the presence of 5% CO_2 at 37°C. HCV (human urothelial cells) were cultured in Eagle's MEM in the presence of 7% (v/v) fetal calf serum and penicillin/streptomycin, and cultured as described above.

2.3. Transfection

Two constructs were used for transfection experiments: one encoding a GFP–calretinin fusion protein and a second encoding only GFP. Both constructs were under the control of the CMV promoter [24]. The plasmids were purified from *Escherichia coli* (DH5α) using a QIAGEN Plasmid kit according to the manufacturer's protocols. Before transfection, cells were trypsinized and resuspended in the culture medium. About 1×10^6 cells and 40 μg of DNA were mixed and incubated on ice for 10 min. Electroporation was then performed in 0.4-mm cuvettes using a BioRad apparatus at 350 V, 500 μFD capacitance, and the cells were again incubated on ice for 10 min. The cells were then placed on coverslips and cultured for 2 days under normal conditions. GFP fluorescence was analyzed on a Nikon Diaphot inverted microscope to identify the efficiency of transfection.

The transfection of HCV cells was performed using lipofectamine according to the manufacturer's protocols. Stable clones were selected using the antibiotic G-418 (500 μg/ml). The transfected cells were cultured under the conditions described above. GFP fluorescence was analyzed on an inverted microscope.

2.4. Measurement of the $[Ca^{2+}]_i$

The cytoplasmic level of calcium was examined using a video imaging system (MagiCal, Applied Imaging, data processing using Tardis V8.0 (Joyce Loebl)) in single glioma C6 cells as described by Barańska et al. [25]. The cells for these experiments were placed on glass coverslips and cultured for 2 days under normal conditions before analysis. Next, cells were washed once with PBS and once with a buffer containing: 137 mM NaCl, 2.7 mM KCl, 1 mM

Na_2HPO_3, 25 mM glucose, 20 mM Hepes (pH 7.4), 1 mM $MgCl_2$, 1% (v/v) bovine serum albumin and 2 mM $CaCl_2$. Cells were then incubated at 37°C in the above buffer with 1 μM fura-2 AM for 30 min. After incubation, the cells were washed three times with the same buffer solution but without fura-2. Coverslips were mounted in a chamber over a Nikon Diaphot inverted stage microscope (40× oil-immersion objective lens, at 37°C). All substances added during subsequent experiments (Ca^{2+}-ionophore, EGTA, thapsigargin or ADP) were dissolved in the above buffer. The cells were alternatively illuminated with 340 and 380 nm light wavelengths from a xenon lamp. The emitted light was passed through a 510 nm barrier filter into an image-intensified camera (Extended ISIS, Photonic Science). The 340 nm and 380 nm images were software averaged and captured every 2.85 s. The 340 nm and 380 nm signals were examined for real changes in $[Ca^{2+}]_i$. Ratio (R) values were converted to an estimate of $[Ca^{2+}]_i$ using the Grynkiewicz formula [26]: $[Ca^{2+}]_i = K_d \beta (R-R_{min})/(R_{max}-R)$. An intracellular calibration was carried out by the addition of 2 μM ionomycin to glioma C6 cells placed in a solution containing 2 mM Ca^{2+} (R_{max}) and in 5 mM EGTA (R_{min}). The β value of 4.5 and K_d of 224 nM was assumed. Positive transfectants containing GFP were identified before $[Ca^{2+}]_i$ measurements using a 490 nm filter.

2.5. Ca^{2+} binding by GFP–CR fusion protein

A protein fraction containing the GFP–CR fusion protein was obtained from stable transfected HCV cells. These cells were chosen because they yielded a higher amount of GFP–CR than transiently transfected glioma C6 cells (with an average efficiency of about 6%). Cells were homogenized with buffer (50 mM Tris–HCl, 20 mM NaCl, 1 mM EGTA, pH 8.0) and sonicated. Proteins present in the 18 000×g supernatant were separated by SDS–polyacrylamide (15%)-gel electrophoresis (PAGE) and transferred to nitrocellulose in a Bio-Rad Trans-Blot apparatus at 200 mA for 1.5 h at room temperature with a transfer buffer containing 192 mM glycine, 25 mM Tris, 0.1% SDS and 20% methanol (v/v). After protein transfer, the nitrocellulose filter was washed (3×20 min) in a buffer containing 60 mM KCl, 10 mM imidazole/HCl and 5 mM $MgCl_2$, pH 6.8. The nitrocellulose filter was then incubated for 10 min with 0.1 mCi of $^{45}CaCl_2$ (Amersham) in 20 ml of the above buffer as described by Maruyama [27]. Subsequently, the nitrocellulose was washed with 50% ethanol (v/v) for 5 min and dried out. Autoradiographs were produced by exposure for 48–72 h. For the immunodetection of CR, the nitrocellulose filter was washed in TBS (2×10 min) and blocked by 3% BSA for 12 h, 37°C. The nitrocellulose filter was then incubated with anti-calretinin serum (1:3000, NO6) for 2 h and then with anti-rabbit IgG conjugated with horseradish peroxidase (1:1000). Staining was performed using the chloronaphthol reaction.

2.6. Measurement of CR concentration expressed in transfected cells

After transfection, the total number of cells and positive transfectants were counted. The cells were then trypsinized, washed once with PBS, homogenized in a buffer containing 50 mM Tris–HCl, 20 mM NaCl, 1 mM EGTA (pH 8.0), and sonicated. 10 μg of soluble proteins present in the 18 000×g supernatant were separated by SDS–polyacrylamide 10%-gel electrophoresis and transferred to nitrocellulose in a Bio-Rad Trans-Blot apparatus at 200 mA for 1.5 h with a transfer buffer containing 192 mM glycine, 25 mM Tris, 0.1% SDS and 20% methanol (v/v). The nitrocellulose filter was washed in TBS (2×10 min) and blocked by 3% BSA for 12 h, 37°C and then incubated with anti-calretinin serum (1:5000, NO6) for 2 h, and finally with anti-rabbit IgG conjugated with horseradish peroxidase for 1 h (1:1000, Sigma). The CR antibody binding was estimated on films by optical density measurements (densitometer Molecular Dynamics) of the bands developed after reaction with chemiluminescent substrates (KPL). Different time exposures from two independent blots were used for densitometric analysis. As a quantitative standard, recombinant CR purified from *Escherichia coli* [28] was used to estimate the concentration of GFP–CR expressed in transfected cells.

3. Results

3.1. Transfection of glioma C6 cells

To analyze the effects of CR's presence on the properties of glioma C6 cells we transfected them with a plasmid containing the GFP and CR coding regions. Transfected cells were identified by GFP fluorescence, which was visible about 24 h after electroporation (Fig. 1B). Different methods of transfection were investigated. The most efficient transfection was obtained by electroporation with an average efficiency of about 6% (range 3–15%) (as described in Section 2) and this was used throughout the studies reported here. Transfection using liposomes was lethal for cells and transfection by calcium phosphate gave a low efficiency (~1%). Transfected cells were used 48 h after electroporation.

3.2. GFP fluorescence does not interfere with fura-2 fluorescence

The first experiments were designed to determine whether the fluorescence of the S65T mutant of GFP interferes with fura-2 fluorescence. We found that cells containing GFP have the same fura-2 fluorescence as untransfected ones (Fig. 1A,B). Therefore, the use of the S65T mutant of GFP as a marker of successful transfection is compatible with the use of fura-2 to measure intracellular $[Ca^{2+}]$.

3.3. Intracellular $[Ca^{2+}]$ in cells expressing GFP

We examined whether the presence of GFP affects $[Ca^{2+}]_i$ in glioma C6 cells. The changes of $[Ca^{2+}]_i$ in cells expressing GFP and in untransfected cells were compared to each other, both before and after stimulation with ionomycin, ADP and thapsigargin. All the analyzed untransfected cells (82) and the cells expressing GFP (56 cells analyzed) had a similar average basal $[Ca^{2+}]_i$. After the addition of 4 μM ionomycin, all analyzed untransfected cells (23) showed a very similar increases in $[Ca^{2+}]_i$ to those observed in cells expressing GFP (19 cells). Fig. 2A shows the results in which the $[Ca^{2+}]_i$ values for all the analyzed cells were averaged.

After stimulation with 10 μM ADP, 25 untransfected cells out of 33 analyzed (8 did not react)

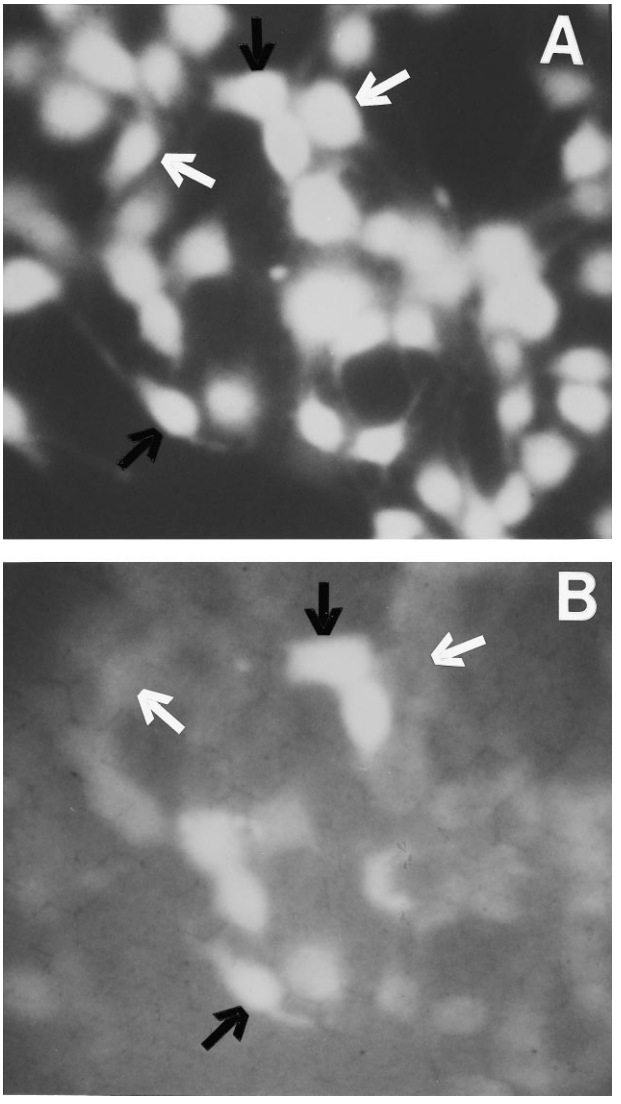

Fig. 1. Glioma C6 cells transfected with the GFP vector. (A) Fura-2 fluorescence (black arrows show transfected cells, white arrows show untransfected cells). (B) GFP control vector fluorescence, with the same field of observation (arrows show the same cells as indicated in A). ×400.

showed a similar increase in the $[Ca^{2+}]_i$ as 19 cells out of 23 analyzed expressing GFP (4 did not react). Fig. 3A shows the results of a single experiment in which the $[Ca^{2+}]_i$ values for analyzed cells (4 transfected and 2 untransfected) were averaged.

After stimulation with 200 nM thapsigargin, 26 untransfected cells showed a similar increase of $[Ca^{2+}]_i$ as 14 cells (all cells reacted) expressing GFP. Fig. 4A shows the results of a single experiment in which the $[Ca^{2+}]_i$ values for analyzed cells (3 transfected and 5 untransfected) were averaged. All

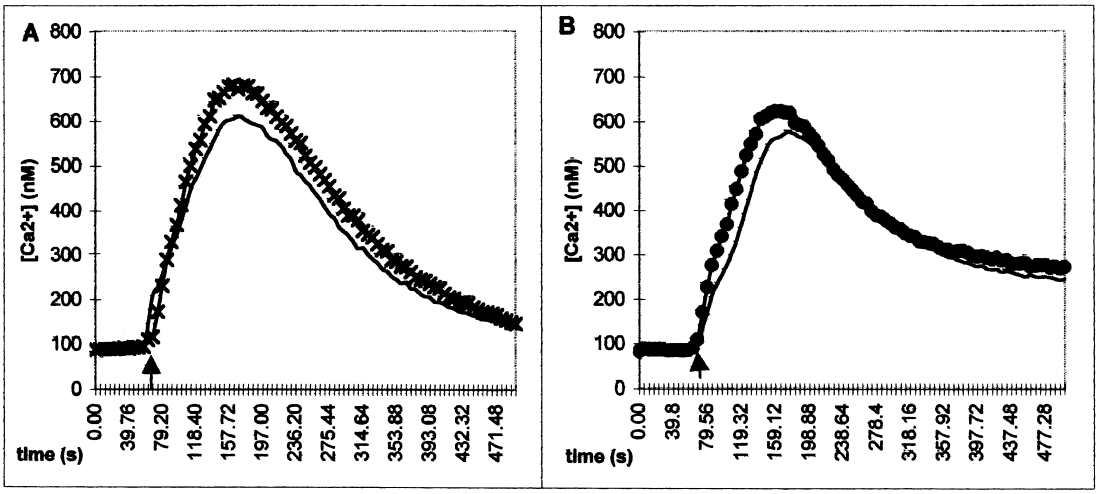

Fig. 2. Changes in $[Ca^{2+}]_i$ in glioma C6 cells stimulated with 4 μM ionomycin. (A) cells ($n=19$) transfected with the GFP control vector (shown with crosses) versus untransfected ones ($n=23$). (B) Cells transfected with GFP–CR ($n=20$) (shown with closed circles) versus untransfected ones ($n=48$). Arrows show when ionomycin was added.

the results suggest that neither GFP expression, nor the transfection process per se, affects the $[Ca^{2+}]_i$ in glioma C6 cells.

3.4. Intracellular $[Ca^{2+}]$ in cells expressing GFP–CR fusion protein

The changes in $[Ca^{2+}]_i$ in cells expressing GFP–CR and untransfected cells were compared with each other, first before and then after stimulation with ionomycin, ADP and thapsigargin. Untransfected cells (85 analyzed) had a similar average basal $[Ca^{2+}]_i$ level as cells expressing GFP–CR (63 analyzed). After the addition of 4 μM ionomycin, all untransfected cells that were analyzed (48 cells) showed a very similar increase in $[Ca^{2+}]_i$ to cells expressing GFP–CR (20 cells). Fig. 2B shows the results in which $[Ca^{2+}]_i$ values for all analyzed cells were averaged.

After stimulation with 10 μM ADP, 18 from 20 untransfected cells showed an identical increase in $[Ca^{2+}]_i$ as was observed in 19 cells from 20 analyzed

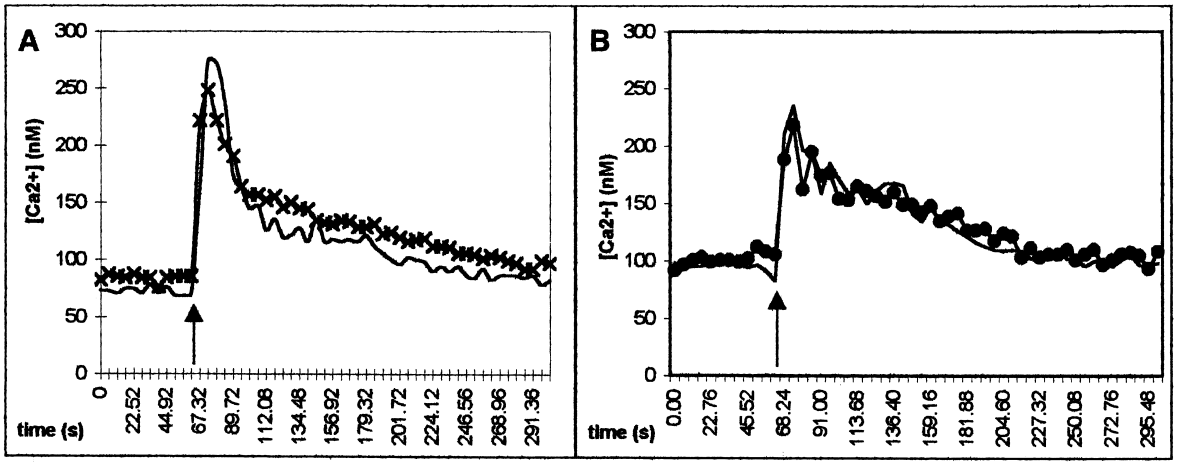

Fig. 3. Changes in $[Ca^{2+}]_i$ in glioma C6 cells stimulated with 10 μM ADP. (A) cells ($n=4$) transfected with GFP control vector (shown with crosses) versus untransfected ones ($n=2$). (B) Cells transfected with GFP–CR ($n=3$) (shown with closed circles) versus untransfected ones ($n=5$). Arrows show when ADP was added.

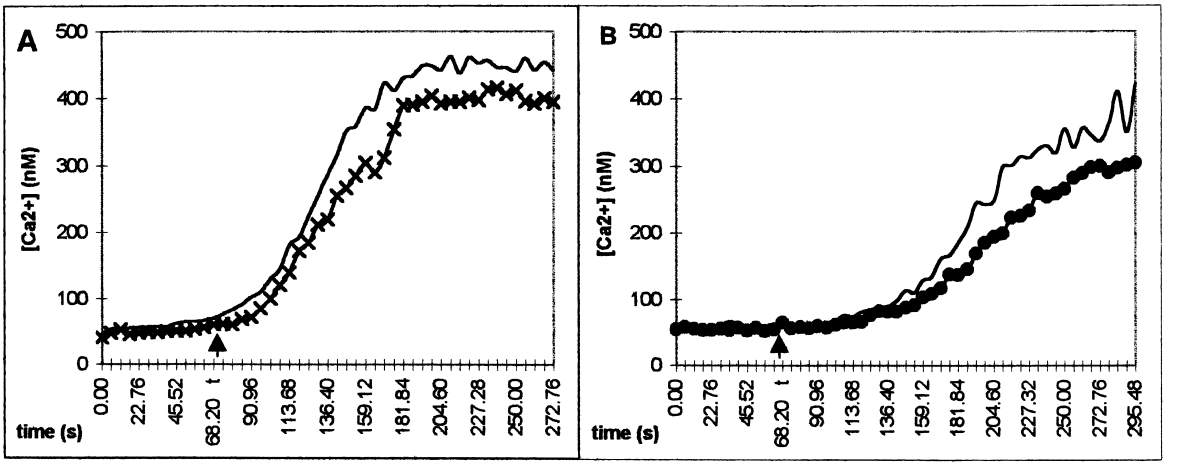

Fig. 4. Changes in $[Ca^{2+}]_i$ in glioma C6 cells stimulated with 200 nM thapsigargin. (A) Cells ($n=3$) transfected with GFP control vectors (shown with crosses) versus untransfected ones ($n=5$). (B) Cells transfected with GFP–CR ($n=4$) (shown with closed circles) versus untransfected ones ($n=4$). Arrows show when thapsigargin was added.

(1 did not react) expressing GFP–CR. Fig. 3B shows the results of a single experiment in which the $[Ca^{2+}]$ values for analyzed cells (3 transfected and 5 untransfected) were averaged.

After stimulation with 200 nM thapsigargin, 17 untransfected cells showed a similar increase in $[Ca^{2+}]_i$ as observed in 23 cells expressing GFP–CR (all cells reacted). Fig. 4B shows the results of a single experiment in which the $[Ca^{2+}]$ values for analyzed cells (4 transfected and 4 untransfected) were averaged. All the results indicate that GFP–CR does not affect the $[Ca^{2+}]_i$ in these cells, either before or after activation.

3.5. Estimation of CR concentration in transfected cells

The protein extract of cells expressing GFP–CR was resolved by SDS–PAGE and blotted onto nitrocellulose to estimate the concentration of the fusion protein. In neighboring wells, known amounts of recombinant CR were run as a quantitative standard. The densitometric analysis of bands containing known amounts of CR was used to obtain a calibration curve from which the amount of GFP–CR was calculated (Fig. 5). It was found that the CR concentration in stable transfected HCV cells was 0.009 pg per cell and in transient transfected glioma C6 cells was about 0.028 pg per cell, assuming an equal expression level in all cells transfected with 15% efficiency. However, 4% of cells showed brighter GFP fluorescence than the other transfected cells and only those cells were used for Ca^{2+} imaging experiments. Based on the results by Staub et al. that the volume of glioma C6 cells is about 900 μm^3 [29], the concentration of CR is estimated to be at about 1.0 μM if all transfected cells are accounted and assumed to have an equal level of GFP–CR. The bright cells

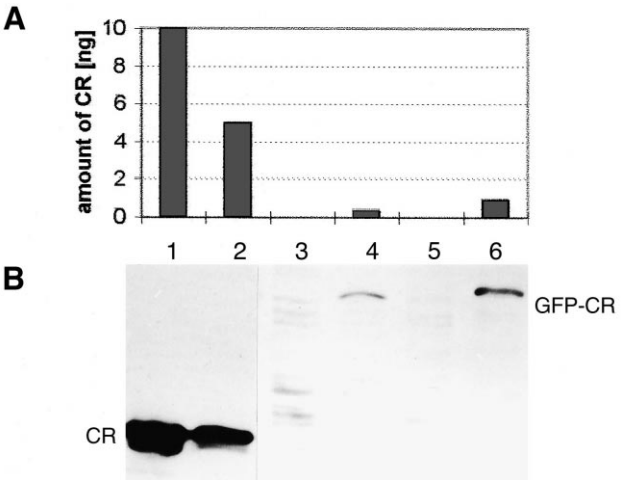

Fig. 5. Estimation of CR content in protein extracts of transfected cells using immunoblotting. (A) Densitometric analysis of the blots. (B) Film exposed after chemiluminescent reaction of the blot. 1, 10 ng of recombinant CR; 2, 5 ng of CR; 3, 10 μg of untransfected glioma C6 cells protein extract; 4, 10 μg of GFP–CR transfected glioma C6 cells protein extract; 5, 10 μg of untransfected HCV cells protein extract; 6, 10 μg of GFP–CR transfected HCV cells protein extract.

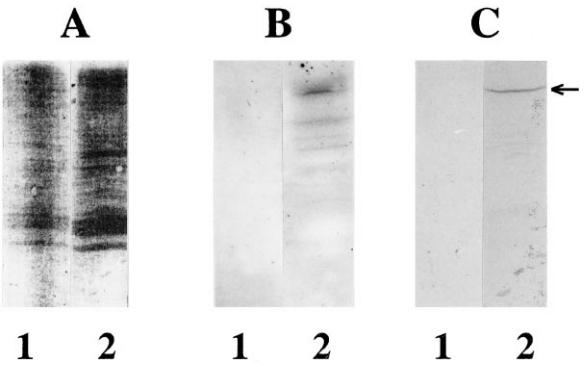

Fig. 6. Ca^{2+} binding by GFP–CR fusion protein. (A) Protein staining with Ponceau Red. (B) Autoradiogram with $^{45}Ca^{2+}$-binding on the same blot. (C) Western blot with anti-CR staining of the same blot (GFP–CR is indicated with arrow). 1, supernatant of control HCV cell extract after centrifugation at 18 000×*g*; 2, supernatant of GFP–CR transfected HCV cell extract after centrifugation at 18 000×*g*.

used for Ca^{2+} imaging experiments had a higher level of GFP–CR but this is difficult to estimate accurately.

3.6. *Ca^{2+} binding by GFP–CR fusion protein*

It was earlier shown that a glutathione-*S*-transferase–CR fusion protein exhibits a Ca^{2+}-binding ability similar to that of recombinant CR [28]. The results in Fig. 6 confirm that CR is able to bind Ca^{2+} as a fusion product with GFP. Therefore, the lack of effects of GFP–CR on the Ca^{2+} level cannot be explained by an inability of the GFP–CR fusion protein to bind Ca^{2+}. Rather, it suggests that CR is unable to significantly affect Ca^{2+} homeostasis in transfected glioma C6 cells.

4. Discussion

As reviewed in the Section 1, several studies suggest that the presence of CR correlates with an increased survival ability of neurons under pathological conditions which are connected with increased intracellular levels of Ca^{2+} [10–12,16]. However, not all studies confirm such a relationship. It has often been postulated that CR does not act as Ca^{2+} buffer and protect neurons against Ca^{2+}-overload [20–23]. As CR-positive cells and CR-negative cells were maintained under the same conditions throughout the experiments, it was possible to directly and simultaneously compare their properties. We found that neither the basic $[Ca^{2+}]_i$, nor activated levels, were affected by CR's presence (as part of a fusion protein) in transfected cells despite CR's ability to bind Ca^{2+}, as shown in this work and by Strauss et al. [28]. The apparent lack of Ca^{2+} buffering by CR supports our earlier work showing that cells overexpressing CR do not show better survival rates when treated with Ca^{2+}-ionophores [24]. Thus, our present results support the hypothesis that CR's function is unlikely to be an intracellular Ca^{2+} buffer [18]. However, the results of the present work are in contrast to the meeting report by Fellay et al., who observed a Ca^{2+} buffering effect with CR overexpression [30]. This discrepancy is difficult to explain but some suggestions can be proposed. The different results could be explained by the different methods of transfection used, its prevalence (stable versus transient transfection), the different cell lines used in the experiments and different agents used to induce the Ca^{2+} signal (those mobilizing Ca^{2+} from the endoplasmic reticulum, or those which cause a Ca^{2+} influx from the extracellular space). It is possible that the effect of CR can only be seen in those cell lines in which CR and its targets are present. In different cell lines the level of expression of the recombinant protein may vary and a different compartmentalization might affect the ability of the protein to react to $[Ca^{2+}]$ changes. Such explanations were provided by Braunewell et al., who found a similar $[Ca^{2+}]_i$ in both visinin-like protein transfected and untransfected glioma C6 cell lines [31]. Compartmentalization of Ca^{2+} pools and their different mobilization, depending on the type of stimuli, is a likely explanation for the different effect of serum versus Ca^{2+} ionophores on stable cell lines expressing calbindin D28k [32].

To buffer $[Ca^{2+}]_i$ from 0.7 μM (achieved by ionomycin) to, for instance, 0.5 μM, it would be enough to have at least 0.15 μM of intracellular CR (assuming four calcium ions bound). We estimate the level of GFP–CR in glioma C6 cells to be at least 1 μM (0.028 pg per cell). The endogenous concentration of CR in neurons is unknown but it was reported that the homologous protein, calbindin D28k, is present at 0.05 pg per Purkinje cell in the rat cerebellum [33]. If the endogenous CR concentration is similar to

that of calbindin D28k then the concentration of GFP–CR is similar to the CR concentration in neurons. Therefore, we believe that the level of expressed GFP–CR in glioma C6 cells is either within the physiological concentration of endogenous CR, or higher. If so, CR should act as a Ca^{2+} buffer in our experimental system. However, even this level of CR does not affect an activated level of [Ca^{2+}] reaching 700 nM (Fig. 2). An unlikely explanation is that the presence of GFP decreases the ability of CR to bind Ca^{2+}; GST in a fusion product with CR seems not to have such an effect [28].

Is CR, then, a general intracellular [Ca^{2+}] buffering protein? We believe that results of this work, and of our earlier work, do not support such a hypothesis. However, many authors propose that this is the case and claim that this is the function of this protein. If the role of CR is to buffer [Ca^{2+}], in other words to perform a crucial function for neuron survival, it is difficult to explain the results in which mice lacking calretinin were studied [34]. First, the brain development appeared to be normal in these mice and there was no evidence the for up-regulation of other calcium binding proteins. Second, the lack of CR expression did not affect motor coordination while the lack of calbindin D28k significantly impaired motor coordination [35], [34]. Why would two similar 'buffer-proteins' behave so differently? Third, CR deficient mice showed no alteration in basal synaptic transmission, whereas long-term potentiation was impaired in the dentate gyrus. These results, as well as the published results on the possible neuroprotection provided by CR, might be explained if CR is involved in Ca^{2+} modulation of specific signaling pathways rather than in overall general intracellular [Ca^{2+}] buffering. Therefore, the term 'Ca^{2+} buffer' for CR seems to be justified only to distinguish it from proteins called 'Ca^{2+} sensors' such as calmodulin. It seems more appropriate to name CR a 'Ca^{2+} modulator' to distinguish it from genuine Ca^{2+} buffers such as parvalbumin or calbindin D9k and to more accurately describe its function.

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References

[1] J.H. Rogers, Calretinin: a gene for a novel calcium-binding protein expressed principally in neurons, J. Cell Biol. 105 (1987) 1343–1353. [published erratum appears in J. Cell Biol., 110(5) (1990) 1845]

[2] M. Parmentier, A. Lefort, Structure of the human brain calcium-binding protein calretinin and its expression in bacteria, Eur. J. Biochem. 196 (1991) 79–85.

[3] K.I. Strauss, D.M. Jacobowitz, Nucleotide sequence of rat calretinin cDNA, Neurochem. Int. 22 (1993) 541–546.

[4] C.J. Dechesne, L. Winsky, H.N. Kim, G. Goping, T.D. Vu, R.J. Wenthold, D.M. Jacobowitz, Identification and ultrastructural localization of a calretinin-like calcium-binding protein (protein 10) in the guinea pig and rat inner ear, Brain Res. 560 (1991) 139–148.

[5] L. Winsky, J. Kuznicki, Distribution of calretinin, calbindin D28k, and parvalbumin in subcellular fractions of rat cerebellum: effects of calcium, J. Neurochem. 65 (1995) 381–388.

[6] M.J. Hubbard, N.J. McHugh, Calbindin28kDa and calbindin30kDa (calretinin) are substantially localised in the particulate fraction of rat brain, FEBS Lett. 374 (1995) 333–337.

[7] L. Winsky, H. Nakata, B.M. Martin, D.M. Jacobowitz, Isolation, partial amino acid sequence, and immunohistochemical localization of a brain-specific calcium-binding protein, Proc. Natl. Acad. Sci. USA 86 (1989) 10139–10143.

[8] V. Gotzos, B. Schwaller, N. Hetzel, M. Bustos-Castillo, M.R. Celio, Expression of the calcium binding protein calretinin in WiDr cells and its correlation to their cell cycle, Exp. Cell. Res. 202 (1992) 292–302.

[9] V. Gotzos, B. Schwaller, J.C. Gander, M. Bustos-Castillo, M.R. Celio, Heterogeneity of expression of the calcium-binding protein calretinin in human colonic cancer cell lines, Anticancer Res. 16 (1996) 3491–3498.

[10] W. Lukas, K.A. Jones, Cortical neurons containing calretinin are selectively resistant to calcium overload and excitotoxicity in vitro, Neuroscience 61 (1994) 307–316.

[11] D.M. Vogt Weisenhorn, E. Weruaga-Prieto, M.R. Celio, Calretinin-immunoreactivity in organotypic cultures of the rat cerebral cortex: effects of serum deprivation, Exp. Brain Res. 108 (1996) 101–112.

[12] C.J. Pike, C.W. Cotman, Calretinin-immunoreactive neurons are resistant to beta-amyloid toxicity in vitro, Brain Res. 671 (1995) 293–298.

[13] A. Mouatt-Prigent, Y. Agid, E.C. Hirsch, Does the calcium binding protein calretinin protect dopaminergic neurons against degeneration in Parkinson's disease?, Brain Res. 668 (1994) 62–70.
[14] P.R. Hof, E.A. Nimchinsky, M.R. Celio, C. Bouras, J.H. Morrison, Calretinin-immunoreactive neocortical interneurons are unaffected in Alzheimer's disease, Neurosci. Lett. 152 (1993) 145–148.
[15] M. Fonseca, E. Soriano, Calretinin-immunoreactive neurons in the normal human temporal cortex and in Alzheimer's disease, Brain Res. 691 (1995) 83–91.
[16] K.R. Isaacs, M.E. Wolpoe, D.M. Jacobowitz, Calretinin-immunoreactive dopaminergic neurons from embryonic rat mesencephalon are resistant to levodopa-induced neurotoxicity, Exp. Neurol. 146 (1997) 25–32.
[17] J. Kuznicki, K.I. Strauss, D.M. Jacobowitz, Conformational changes and calcium binding by calretinin and its recombinant fragments containing different sets of EF hand motifs, Biochemistry 34 (1995) 15389–15394.
[18] J. Kuznicki, T.L. Wang, B.M. Martin, L. Winsky, D.M. Jacobowitz, Localization of Ca^{2+}-dependent conformational changes of calretinin by limited tryptic proteolysis, Biochem. J. 308 (1995) 607–612.
[19] B. Schwaller, I. Durussel, D. Jermann, B. Herrmann, J.A. Cox, Comparison of the Ca^{2+}-binding properties of human recombinant calretinin-22k and calretinin, J. Biol. Chem. 272 (1997) 29663–29671.
[20] V. Mockel, G. Fischer, Vulnerability to excitotoxic stimuli of cultured rat hippocampal neurons containing the calcium-binding proteins calretinin and calbindin D28K, Brain Res. 648 (1994) 109–120.
[21] J.P. Brion, A. Resibois, A subset of calretinin-positive neurons are abnormal in Alzheimer's disease, Acta Neuropathol. (Berl.) 88 (1994) 33–43.
[22] T.F. Freund, Z. Magloczky, Early degeneration of calretinin-containing neurons in the rat hippocampus after ischemia, Neuroscience 56 (1993) 581–596.
[23] K. Yamada, S. Goto, T. Oyama, M. Yoshikawa, S. Nagahiro, Y. Ushio, Striatal cells containing the Ca^{2+}-binding protein calretinin (protein 10) in ischemia-induced neuronal injury, Acta Neuropathol. (Berl.) 89 (1995) 172–177.
[24] J. Kuznicki, K.R. Isaacs, D.M. Jacobowitz, The expression of calretinin in transfected PC12 cells provides no protection against Ca^{2+}-overload or trophic factor deprivation, Biochim. Biophys. Acta 1313 (1996) 194–200.
[25] J. Baranska, V. Chaban, M. Czarny, P. Sabala, Changes in Ca^{2+} concentration in phorbol ester and thapsigargin treated glioma C6 cells. The role of protein kinase C in regulation of Ca^{2+} entry, Cell Calcium 17 (1995) 207–215.
[26] G. Grynkiewicz, M. Poenie, R.Y. Tsien, A new generation of Ca^{2+} indicators with greatly improved fluorescence properties, J. Biol. Chem. 260 (1985) 3440–3450.
[27] K. Maruyama, T. Mikawa, S. Ebashi, Detection of calcium binding proteins by ^{45}Ca autoradiography on nitrocellulose membrane after sodium dodecyl sulfate gel electrophoresis, J. Biochem. (Tokyo) 95 (1984) 511–519.
[28] K.I. Strauss, J. Kuznicki, L. Winsky, D.M. Jacobowitz, Expression and rapid purification of recombinant rat calretinin: similarity to native rat calretinin, Protein Expr. Purif. 5 (1994) 187–191.
[29] F. Staub, J. Peters, N. Plesnila, R.C. Chang, A. Baethmann, Swelling and damage of glial cells by lactacidosis and glutamate: effect of alpha-trinositol, Brain Res. 766 (1997) 285–288.
[30] B.S. Fellay, B. Schneider, B. Gotzos V, M.R. Celio, in: Fourth European Symposium on Calcium Binding Proteins in Normal and Transformed Cells, Cassa di Risparmio di Firenze, Perugia, Italy, 1996, p. P49.
[31] K.H. Braunewell, C. Spilker, T. Behnisch, E.D. Gundelfinger, The neuronal calcium-sensor protein VILIP modulates cyclic AMP accumulation in stably transfected C6 glioma cells: amino-terminal myristoylation determines functional activity, J. Neurochem. 68 (1997) 2129–2139.
[32] E.M. Muir, D.E. Lawson, F.V. Sepulveda, J.A. O'Brien, M. Harding, Calbindin D28K expression in transfected mouse NIH3T3 cells, Cell Calcium 14 (1993) 349–358.
[33] N. Kurobe, Y. Inaguma, H. Shinohara, R. Semba, T. Inagaki, K. Kato, Developmental and age-dependent changes of 28-kDa calbindin-D in the central nervous tissue determined with a sensitive immunoassay method, J. Neurochem. 58 (1992) 128–134.
[34] S. Schurmans, S.N. Schiffmann, H. Gurden, M. Lemaire, H.P. Lipp, V. Schwam, R. Pochet, A. Imperato, G.A. Bohme, M. Parmentier, D.B. Arnold, N. Heintz, Impaired long-term potentiation induction in dentate gyrus of calretinin-deficient mice. A calcium responsive element that regulates expression of two calcium binding proteins in Purkinje cells, Proc. Natl. Acad. Sci. USA 94 (1997) 10415–10420.
[35] M.S. Airaksinen, J. Eilers, O. Garaschuk, H. Thoenen, A. Konnerth, M. Meyer, Ataxia and altered dendritic calcium signaling in mice carrying a targeted null mutation of the calbindin D28k gene, Proc. Natl. Acad. Sci. USA 94 (1997) 1488–1493.